



The *Helicobacter pylori* adhesin protein HopQ exploits the dimer interface of human CEACAMs to facilitate translocation of the oncoprotein CagA

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Abstract

Helicobacter pylori infects half of the world's population, and strains that encode the *cag* type IV secretion system for injection of the oncoprotein CagA into host gastric epithelial cells are associated with elevated levels of cancer. CagA translocation into host cells is dependent on interactions between the *H. pylori* adhesin protein HopQ and human CEACAMs. Here, we present high-resolution structures of several HopQ-CEACAM complexes and CEACAMs in their monomeric and dimeric forms establishing that HopQ uses a coupled folding and binding mechanism to engage the canonical CEACAM dimerization interface for CEACAM recognition. By combining mutagenesis with biophysical and functional analyses, we show that the modes of CEACAM recognition by HopQ and CEACAMs themselves are starkly different. Our data describe precise molecular mechanisms by which microbes exploit host CEACAMs for infection and enable future development of novel oncoprotein translocation inhibitors and *H. pylori*-specific antimicrobial agents.

Keywords CagA; CEACAM; *Helicobacter pylori*; HopQ; X-ray crystallography

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Microbiology, Virology & Host Pathogen Interaction; Structural Biology

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Introduction

Helicobacter pylori is a gastric pathogen that infects more than half of the human population worldwide and is a major cause of

gastrointestinal diseases, such as chronic active gastritis and peptic ulcers, as well as stomach cancers including mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Peek & Blaser, 2002; Suerbaum & Michetti, 2002). Certain *H. pylori* strains harbor the 37-kilobase *cytotoxin-associated gene-pathogenicity island* (*cag*-PAI) that encodes numerous Cag proteins that collectively form a membrane-spanning type IV secretion system (T4SS), as well as the effector protein CagA. Upon contact with gastric epithelial cells, CagA is translocated through the T4SS into host cells (Odenbreit *et al.*, 2000) prompting a pro-inflammatory response (Fischer *et al.*, 2001; Gorrell *et al.*, 2013). Once inside the host cell, CagA is phosphorylated and subsequently interacts with numerous host cell proteins (Higashi *et al.*, 2002; Tsutsumi *et al.*, 2003; Selbach *et al.*, 2009) to impair tight junctions and cause loss of epithelial cell polarity (Amieva *et al.*, 2003; Saadat *et al.*, 2007), disrupt kinase signaling, and modulate the apoptotic program (Buti *et al.*, 2011). CagA is considered a bacterial oncoprotein (Hatakeyama, 2004) as its presence is correlated with the risk of developing cancer in humans, and it induces gastrointestinal and hematopoietic neoplasms in mice (Ohnishi *et al.*, 2008).

Several host-pathogen interactions are functional contributors to *H. pylori* adherence to human gastric epithelial cells and CagA translocation. The $\alpha_5\beta_1$ -integrin heterodimer is a target for proteins encoded by the *cag*-PAI, including CagA (Kwok *et al.*, 2007; Jimenez-Soto *et al.*, 2009) and CagL (Bonsor *et al.*, 2015b). A family of *H. pylori* adhesin proteins, the *Helicobacter* outer proteins (Hops), bind to both glycans and proteins on the host cell surface. Of these, BabA and SabA bind to the human blood group antigens, Lewis b (Le^b) and sialylated Lewis X (sLe^x), respectively, to facilitate the bacterium's persistence at the gastric mucosa (Ilver *et al.*, 1998; Mahdavi *et al.*, 2002; Linden *et al.*, 2004). Numerous X-ray crystal

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structures of BabA alone and in complex with Le^b carbohydrates (Hage *et al*, 2015; Moonens *et al*, 2016) provide a comprehensive structural basis for glycan recognition by *H. pylori*. Another Hop, HopQ, has been shown to exploit human carcinoembryonic antigen-related cell adhesion molecule (CEACAM) proteins for both host cell adherence and CagA translocation (Javaheri *et al*, 2016; Koniger *et al*, 2016). Although a high-resolution structure of HopQ has been reported (Javaheri *et al*, 2016), it remains unclear how HopQ binds to CEACAMs.

The CEACAM family forms a group of extracellular receptors involved in cell adhesion, differentiation, proliferation, signaling, tumor suppression, and survival (Benchimol *et al*, 1989; Singer *et al*, 2005, 2010; Gray-Owen & Blumberg, 2006; Tchoupa *et al*, 2014). The 12 human CEACAMs are each comprised of: (i) a single N-terminal variable immunoglobulin (IgV) domain that can homo- and/or heterodimerize with themselves or other CEACAMs (Bonsor *et al*, 2015a); (ii) a variable number of constant subtype 2 (IgC2) domains, ranging from zero to six, separating the IgV domain and the plasma membrane anchor, which is either a transmembrane helix or a glycoposphatidylinositol linker; and (iii) for some CEACAMs, an intracellular signaling motif. Beyond their role as cell adhesion and signaling molecules, CEACAMs, in particular CEACAM1, CEACAM5, and CEACAM6, are also targets of diverse pathogenic bacteria, including *Neisseria gonorrhoeae* and *Escherichia coli* (Tchoupa *et al*, 2014). Each of these bacteria has convergently evolved distinct cell surface adhesins to bind CEACAMs and exploit the human mucosa, on which the above-mentioned CEACAMs are expressed on the apical side, as an attachment site for colonization, multiplication, and spread (Virji, 2009); or for endocytosis into epithelial cells and transcytosis through intact epithelial layers (Virji *et al*, 1996; Gray-Owen *et al*, 1997a,b). More recently, CEACAMs have been shown to be exploited by the *H. pylori* adhesin protein HopQ for translocation of the oncoprotein CagA (Javaheri *et al*, 2016; Koniger *et al*, 2016).

Certain bacterial adhesins also bind to CEACAM3, which does not interact with itself or other CEACAM family members. CEACAM3 is expressed on granulocytes and contains a cytoplasmic ITAM motif. Host–pathogen interactions involving CEACAM3 promote phagocytosis and clearance of bacteria such as *N. gonorrhoeae* (McCaw *et al*, 2003; Schmitter *et al*, 2007), a process distinct from the transcytosis across the epithelia supported by other CEACAMs. Through this mechanism, CEACAM3 has been proposed to function as an innate immune receptor for human-restricted pathogens that arose by an evolutionary response in the host to recognize and eliminate CEACAM-coopting bacteria (Pils *et al*, 2008).

Here, we describe the molecular basis by which *H. pylori* HopQ engages human CEACAMs, thereby allowing CagA translocation. We determined X-ray crystal structures of HopQ in complex with both CEACAM1 and CEACAM3 N-terminal dimerization domains, the first high-resolution structures of host–pathogen interactions involving CEACAMs. We find that HopQ recognizes CEACAMs through a coupled folding and binding mechanism and binds at the same site that CEACAMs use to homo- and heterodimerize. Despite this common interface, the molecular mechanisms of HopQ-CEACAM interaction and CEACAM dimerization are entirely distinct.

Results

HopQ exploits the CEACAM dimer interface for binding

We crystallized and determined the 2.7 Å resolution structure of the complex formed by the recombinant extracellular domain of the *H. pylori* P12 strain HopQ and the N-terminal IgV domain of human CEACAM1 (Fig 1A and Table EV1). In the crystal, the complex is comprised of one HopQ molecule bound to one CEACAM1 molecule. CEACAMs, including CEACAM1, form homo- and heterodimers with themselves and other CEACAMs via interactions of their N-terminal IgV domains (Bonsor *et al*, 2015a). From the HopQ-CEACAM1 crystal structure, it is evident that HopQ binds CEACAM1 by exploiting the dimer surface of the latter, thereby prohibiting CEACAM1 dimerization and consistent with a 1:1 binding stoichiometry. The overlap of the CEACAM1 interfaces used for HopQ binding and for dimerization is extensive (Fig 1A) and includes 26 common residues, including Phe29, Gln44, Ile91, and Val96. In order to confirm that HopQ-CEACAM1 forms 1:1 complexes in solution and not just in a crystalline environment, we performed native mass spectrometry on the complex and observed a dominant species at 58.8 kDa, equivalent to one HopQ extracellular domain (46.8 kDa) and one CEACAM IgV domain (12.0 kDa; Fig 1B).

Upon HopQ-CEACAM1 complex formation, a total of 2,020 Å² of surface area is buried, which is 400 Å² more than in the CEACAM1 homodimer (Fedarovich *et al*, 2006). Four loops in HopQ make intermolecular contacts with CEACAM1: the α3-β1 loop (residues 102–111); the β2-α4 loop (residues 134–161); the α5-α6 loop (residues 240–244); and the α7-α8 loop (residues 363–385; Fig 2A). Of these, the β2-α4 loop contributes approximately 60 percent of the HopQ buried surface area in the complex. The β-hairpin motif of the β2-α4 loop (residues 135–138) extends the CEACAM1 GFCC'C" β-sheet (i.e., the β sheet composed of the G, F, C, C', and C" β strands of the CEACAM1 Ig-fold domain) by main-chain hydrogen bonding to residues 95–97 of CEACAM1 (Fig 2B), similar to β-zipper motifs. This places the small α-helix and random coil of the β2-α4 loop that follow the β-hairpin across the face of GFCC'C" sheet, forming extensive van der Waals and hydrogen bonding interactions across all of the CEACAM1 β-strands (Fig 2C). These interactions include several residues that are known to be important for CEACAM1 dimerization including Phe29^{CEACAM1}, Val39^{CEACAM1}, Gln44^{CEACAM1}, Gln89^{CEACAM1}, and Leu95^{CEACAM1} (Fedarovich *et al*, 2006). The majority of hydrogen bonds formed between CEACAM1 and the HopQ β2-α4 loop are from main-chain atoms with only six of the 14 hydrogen bonds from side-chain atoms. Only the most apical of the eleven residues in the HopQ α7-α8 loop contact CEACAM1. Asn371^{HopQ} forms van der Waals contacts with Leu95^{CEACAM1}, and Thr370^{HopQ} forms a hydrogen bond with the main-chain carbonyl of Asp94^{CEACAM1} (Fig 2D). The remaining two HopQ loops, α3-β1 and α5-α6, form minimal interactions with CEACAM1, including van der Waals contacts between Ile102^{HopQ}, Phe29^{CEACAM1}, Tyr106^{HopQ}, Thr56^{CEACAM1}, Pro110^{HopQ}, Pro59^{CEACAM1}/Gly41^{CEACAM1}, and Gly111^{HopQ}-Gly41^{CEACAM1}/Asn42^{CEACAM1} and a single hydrogen bond between the main-chain amine of Ala243^{HopQ} and the main-chain carbonyl of Ser93^{CEACAM1} (Fig 2D).

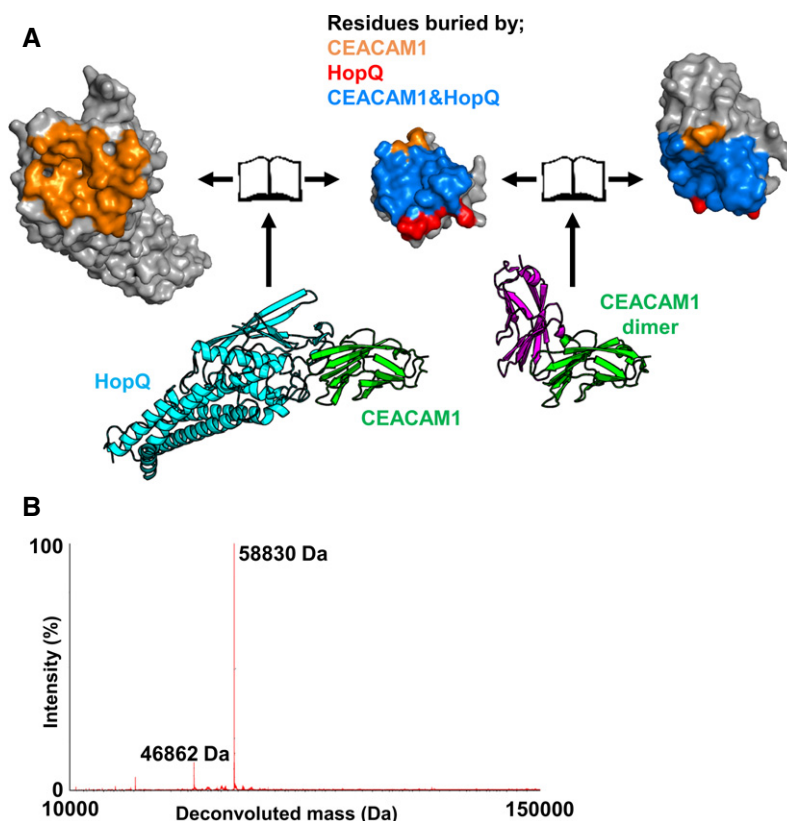


Figure 1. Crystal structure of the HopQ-CEACAM1 complex.

A Bottom left—Crystal structure of HopQ-CEACAM1 (cyan and green, respectively). Bottom right—The CEACAM1 homodimer (pdb code 2gk2) is oriented to overlay with CEACAM1 (green) in the HopQ-CEACAM1 structure. The other half of CEACAM1 dimer is shown as magenta. Both complexes are rendered as a molecular surface representation in an open-book conformation with HopQ (top left), CEACAM1 of both complexes (center), and CEACAM1 of the dimer (top right). Surfaces are colored according to residues buried by just CEACAM1 (orange), just HopQ (red), and by both HopQ and CEACAM1 (blue).

B Deconvolution of raw native mass spectrometry data through maximum entropy displays a predominant population of 58,830 Da (mass of the HopQ-CEACAM1 complex) and a minor population of 46,862 Da (uncomplexed HopQ).

Glycosylation contributes minimally to HopQ-CEACAM interactions

Helicobacter pylori utilizes several adhesin proteins to adhere to host cell surfaces. These adhesins are members of the *Helicobacter* outer membrane protein (Hop) and Hop-related (Hor) gene families, from which HopQ, BabA, and SabA cluster phylogenetically. BabA and SabA are both glycan recognition bacterial adhesins—BabA binds Le^b antigens, while SabA binds sLe^x antigens. HopQ shares sequence identities of 35 and 23% with BabA and SabA, respectively, and the extracellular domains of all three proteins are structurally related, comprising 4 + 3 α helical bundles sprouting from coiled-coil stems formed by their N- and C-termini that link them to their transmembrane domains. An insertion domain that varies in size and sequence for HopQ, BabA, and SabA is found between α helices 4 and 5. While the insertion domain comprises the glycan-binding site for both BabA and SabA (Pang *et al*, 2014; Hage *et al*, 2015; Moonens *et al*, 2016), it is quite distant from the CEACAM-binding site for HopQ (Fig 3A and B).

Despite the abbreviated nature of the HopQ insertion domain, HopQ could potentially bind glycans and the lack of contacts

between the insertion domain and CEACAM1 in our crystal structure could have resulted from our use of an aglycosylated CEACAM1 IgV domain expressed in *E. coli*. The N-terminal IgV domain of CEACAM1 is predicted to contain three N-linked glycosylation sites, Asn70^{CEACAM1}, Asn77^{CEACAM1}, and Asn81^{CEACAM1}, of which Asn70^{CEACAM1} and Asn77^{CEACAM1} are found in the DE and EF loops on the back face of CEACAM1 pointing away from the HopQ insertion domain. Asn81^{CEACAM1} is also found in the EF loop but is facing toward the insertion domain; however, it is positioned more than 30 Å from the insertion domain. While previous studies identified that glycans were not necessary for HopQ binding (Javaheri *et al*, 2016; Koniger *et al*, 2016), only the N-terminal IgV domain was tested. CEACAM1, though, also contains three IgC2 domains that can be glycosylated and could potentially contact the insertion domain. To assess any potential role for glycans in CEACAM1 recognition by HopQ, we expressed the entire ectodomain of CEACAM1 (CEACAM1_{IgV-3IgC2}; inclusive of one IgV and three IgC2 domains) in HEK293 cells in the presence of kifunensine, a mannosidase I inhibitor, to produce CEACAM1 decorated with high-mannose glycans, half of which we treated with EndoA, an endoglycosidase that specifically hydrolyzes high-mannose glycans, leaving only a single

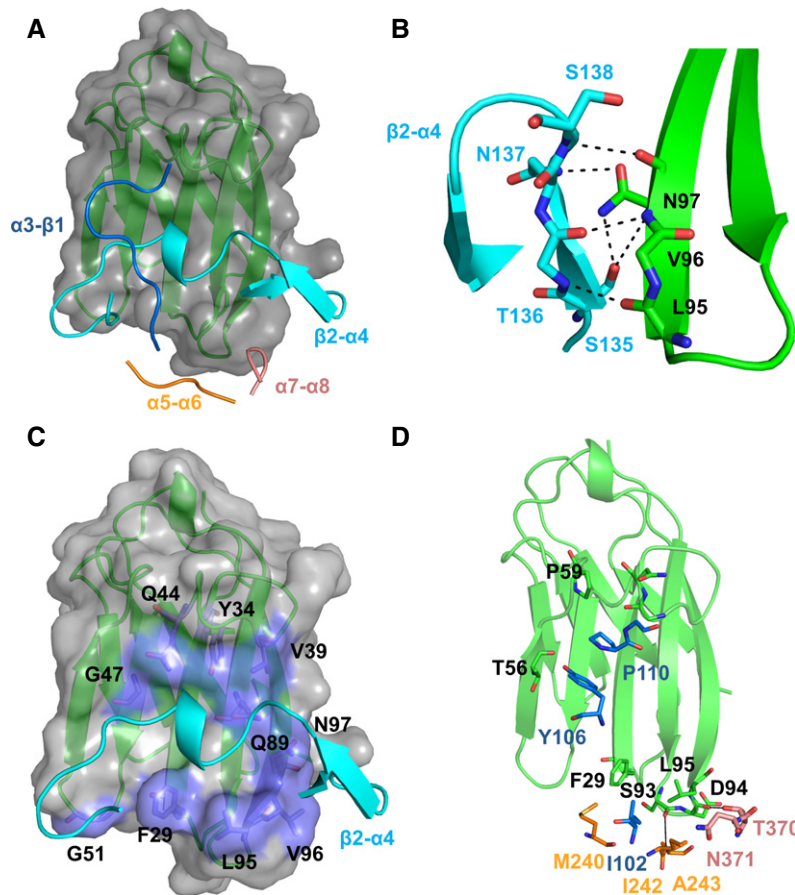


Figure 2. Four loops of HopQ make interactions with CEACAM1.

- A Cartoon representation of CEACAM1 (green) with a molecular surface (gray) bound by the four loops of HopQ: $\alpha 3$ - $\beta 1$ (blue); $\beta 2$ - $\alpha 4$ (cyan); $\alpha 5$ - $\alpha 6$ (yellow); and $\alpha 7$ - $\alpha 8$ (pink).
- B Residues 135–138 of the $\beta 2$ - $\alpha 4$ loop (cyan) form a hydrogen bond network (black lines) to residues 95–97 of CEACAM1 (green).
- C Residues 145–156 of the $\beta 2$ - $\alpha 4$ loop (cyan) make extensive van der Waals contacts (lilac) across all five β -strands of CEACAM1 (green).
- D The $\alpha 3$ - $\beta 1$, $\alpha 5$ - $\alpha 6$, and $\alpha 7$ - $\alpha 8$ loops (blue, yellow, pink, respectively) form several van der Waals contacts and hydrogen bonds (black lines) above and below the $\beta 2$ - $\alpha 4$ loop to CEACAM1 (green).

N-linked N-acetylglucosamine. We also expressed CEACAM1_{IgV-3IgC2} in HEK293 cells in the absence of glycosidase inhibitors in order to produce the full-length CEACAM1 ectodomain decorated predominantly with complex biantennary-type glycans. Using isothermal titration calorimetry (ITC), we measured no differences in binding affinity or thermodynamic parameters for high-mannose and deglycosylated CEACAM1_{IgV-3IgC2} binding to HopQ relative to CEACAM1_{IgV} (Fig 3C–E), indicating that no significant roles in molecular recognition can be ascribed to either CEACAM high-mannose-type glycans or the HopQ insertion domain. However, we observed a 10-fold increase in affinity between HopQ and CEACAM1_{IgV-3IgC2} displaying complex-type glycans caused by both a favorable increase in binding enthalpy and a smaller entropic penalty relative to deglycosylated CEACAM1_{IgV-3IgC2} and its high-mannose-type glycoform (Fig 3F). If complex glycans were binding HopQ, we would expect a larger entropic penalty due to restriction of the CEACAM, suggesting that these glycans may help pre-order CEACAM1 into a HopQ-receptive conformation.

HopQ-CEACAM and BabA-Le^b interactions are affected in opposite ways by pH and redox state

HopQ, BabA, and SabA contain 3, 4, and 2 disulfides, respectively. All three disulfides in HopQ are conserved in BabA, while they do not align with those in SabA. In all three proteins however, the disulfides are observed in loops, constricting conformational sampling of these loops. BabA has been shown to be redox sensitive—the presence of reducing agent substantially weakens its affinity for Le^b and the redox-active compound N-acetylcysteine inhibits Le^b binding, as well as BabA-mediated adherence and inflammation by *H. pylori* (Moonens *et al*, 2016). By conducting ITC binding experiments in the presence of a large molar excess of the reducing agent β -mercaptoethanol, we observed that the reduction in all disulfides in HopQ corresponded to a modest increase in affinity for CEACAM1_{IgV} (Fig 3G). This is in contrast to BabA-Le^b interactions, for which reduction in BabA disulfide bonds prevents Le^b ligand binding (Moonens *et al*, 2016).

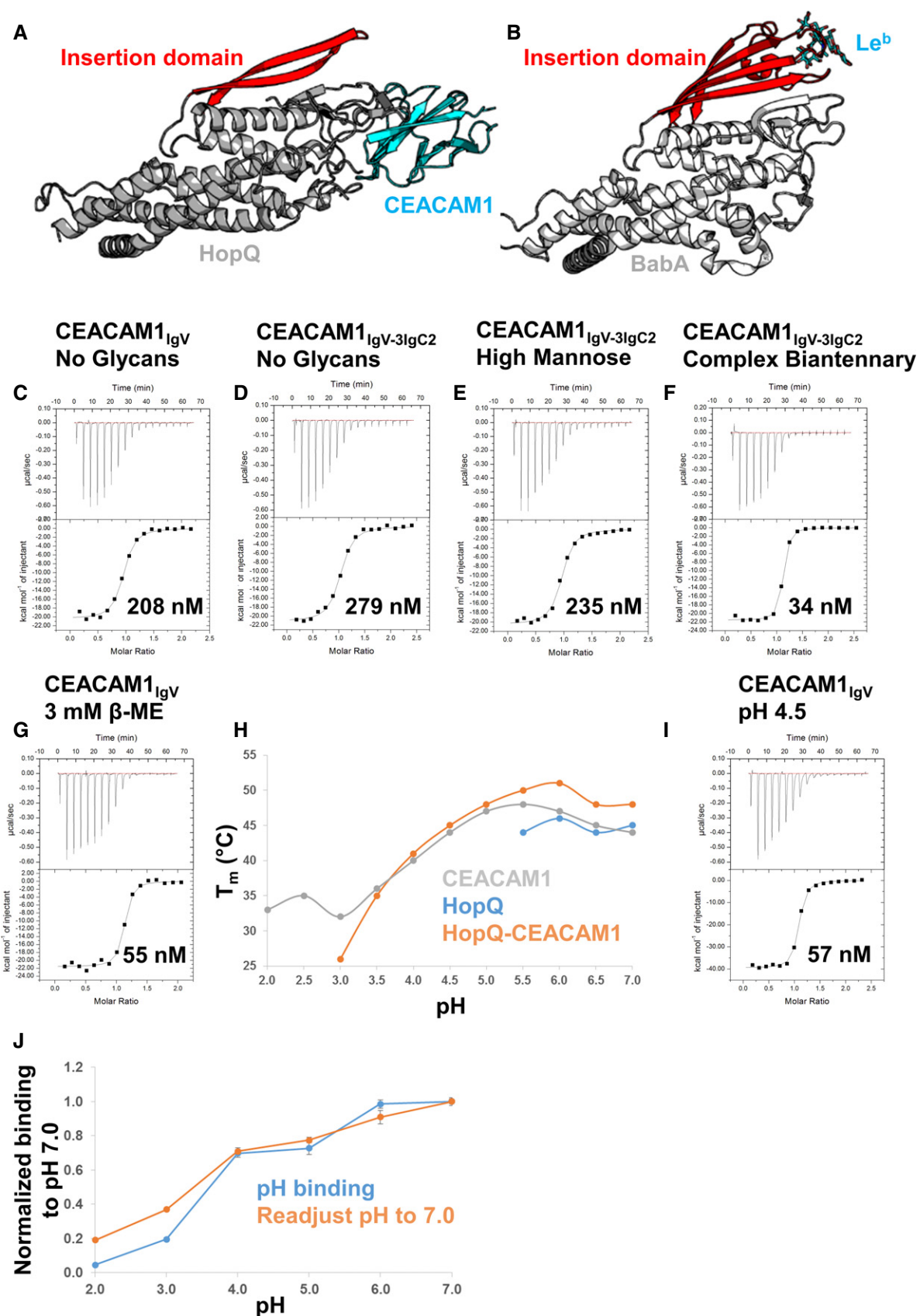


Figure 3.

Figure 3. Characterization of glycans, reduction, and pH on the HopQ-CEACAM1 interaction.

- A Cartoon representation of the HopQ-CEACAM1 complex (gray and cyan, respectively) with the insertion domain of HopQ highlighted in red showing no contacts are made to CEACAM1.
- B Cartoon representation of BabA (pdb code 5f9d, gray). The insertion domain of BabA (red) is in complex with the Le^b heptasaccharide antigen (cyan sticks).
- C–G Isothermal titration calorimetry profiles of HopQ binding to (C) CEACAM1_{IgV} no glycans, (D) CEACAM1_{IgV-3IgC2} no glycans, (E) CEACAM1_{IgV-3IgC2} high-mannose glycans, (F) CEACAM1_{IgV-3IgC2} complex biantennary glycans, and (G) CEACAM1_{IgV} with 3 mM β-mercaptoethanol.
- H Melting temperatures (T_m) of CEACAM1 (gray), HopQ (blue), and the HopQ-CEACAM1 complex (orange) were measured by thermal shift assay at various pHs between pH 2.0 and 7.0.
- I ITC profile of HopQ-CEACAM1_{IgV} at pH 4.5.
- J Acid sensitivity of HopQ binding to CEACAM1 as measured by ELISA at several pHs between 2.0 and 7.0 for 90 min is plotted as the blue line. The orange line shows the recovery of HopQ after 60 min of exposure to the various pHs by readjustment of the pH to 7.0. Two independent experiments were performed on four replicates ($n = 8$). Data were plotted as an average with error bars as standard errors of the mean.

Helicobacter pylori has evolved the ability to survive the pH gradient that exists in the stomach from pH 2.0 in the lumen to pH 7.0 found deep in the gastric mucosal layer close to the epithelial surface. The role of pH in BabA recognition of Le^b antigens has been extensively studied (Bugaytsova *et al*, 2017)—BabA binds Le^b antigens maximally at pH 5.0–6.0 and is observed to have a pH_{50} of ~2.4–3.8 (depending on the *H. pylori* strain); furthermore, when reconditioned to a higher pH after exposure to low pH, BabA recovers 75–100% of its binding capacity to Le^b antigens. Accordingly, we sought to determine the pH responsiveness of HopQ to CEACAM1 and whether HopQ exhibits pH reconditioning. First, we determined the melting temperature (T_m) of CEACAM1_{IgV} and HopQ individually by differential scanning fluorimetry (DSF) at various pH values between 2.0 and 7.0. At low pH, CEACAM1_{IgV} has an estimated T_m of 31°C which increases with pH to a maximum of 47°C between pH 5.0 and 6.0 before decreasing to 43°C at pH 7.0 (Fig 3H). We found that HopQ was less stable than CEACAM1_{IgV} over the same pH range. We observed fluorescence changes occurring only above pH 5.5, with a T_m of approximately 45°C (Fig 3H). Formation of the HopQ-CEACAM1_{IgV} complex led to an increase in T_m of 3–5°C at high pH, but also produced fluorescence changes down to pH 3.0, demonstrating that the HopQ-CEACAM1_{IgV} complex is substantially more stable over a broader pH range than is HopQ alone (Fig 3H). We also performed an ITC binding analysis at pH 4.5 for the CEACAM1_{IgV}-HopQ interaction and observed a threefold increase in affinity ($K_D \sim 60$ nM; Fig 3I). Because ITC experiments at lower pH are not feasible due to the acid-sensitive cell of this instrument, we measured binding of HopQ to a CEACAM1_{IgV}-coated plate at pHs between 2.0 and 7.0 in an ELISA. We observed that HopQ binds CEACAM1 above pH 3.0, with a pH_{50} of 3.5, with minimal binding at pH 2.0 (Fig 3J). Using the same ELISA setup, after incubation of the plate with HopQ at the various pHs, we readjusted the pH to pH 7.0 in order to determine whether CEACAM1 binding by HopQ was recoverable. We found that binding in the reconditioned medium nearly identically mirrors the binding curve of the pH-dependent interaction indicating that, unlike the BabA-Le^b interaction, HopQ binding to CEACAM is not reversible when acidity is decreased (Fig 3J).

HopQ recognizes CEACAMs through a coupled folding and binding mechanism

In the structure of *H. pylori* strain G27 HopQ in the absence of CEACAM reported previously (Javaheri *et al*, 2016), several loops lack sufficient electron density required to model their conformations. Notably, these include the $\beta 2$ - $\alpha 4$ and $\alpha 7$ - $\alpha 8$ loops, both of

which are ordered and form intermolecular contacts in our HopQ-CEACAM1 complex (Fig 4A and B). The amino acid sequences between the HopQ proteins from the *H. pylori* G27 and P12 strains differ by only a single residue in the $\beta 2$ - $\alpha 4$ and three residues in the $\alpha 7$ - $\alpha 8$ loops, with Tyr144^{HopQ-G27} to Ser144^{HopQ-P12}, and Ala364^{HopQ-G27} to Met364^{HopQ-P12}, Ala370^{HopQ-G27} to Thr370^{HopQ-P12}, and Asn376^{HopQ-G27} to Ser376^{HopQ-P12} polymorphisms, respectively (Appendix Fig S1). Only the HopQ residue at position 370 contacts CEACAM1 (see above), forming a hydrogen bond to Asp94^{CEACAM1}.

Conversely, several other structural features that are ordered in the unbound HopQ structure appear disordered in our CEACAM1-bound structure of HopQ, including (i) the $\beta 1$ - $\beta 2$ loop in the middle of the two-stranded β -hairpin structure of the insertion domain and (ii) the coiled-coil domain formed by the N- and C-terminal α -helices, $\alpha 1$ and $\alpha 8$, the latter of which links the HopQ extracellular domain to its β -barrel transmembrane domain (Fig 4A). Neither the $\beta 1$ - $\beta 2$ loop nor the $\alpha 1$ and $\alpha 8$ coiled coil contacts CEACAM1. Beyond these above-mentioned structural differences in the CEACAM-bound and unbound HopQ structures, there are no gross structural changes in HopQ, which is reflected by a low root mean square deviation (RMSD) for C α atoms of 0.60 Å.

The IgV domain of CEACAM1 in the HopQ-CEACAM1 structure exhibits the classic IgV fold, consisting of two β -sheets, ABED and GFCC'C'', the latter of which forms the dimerization interface observed in all CEACAMs. All residues of the CEACAM1 molecule, except for the N-terminal residue, are resolved in the HopQ-CEACAM1 structure. The RMSD of CEACAM1 C α atoms in the complex relative to that of CEACAM1 (Fedarovich *et al*, 2006) is low (0.66 Å) with only the BC, C'C'', and GF loops exhibiting observable deviations in the HopQ-bound structure (Fig 4C). Because HopQ loops that form a substantial part of the HopQ-CEACAM interface adopt a single conformation in the complex, HopQ recognizes CEACAMs through a coupled folding and binding mechanism. At the same time, several structural elements outside of the HopQ-CEACAM interface become disordered upon binding, with unknown, if any, functional consequences.

CEACAM1 dimerization and HopQ-CEACAM1 interaction exhibit distinct energetic landscapes

To gain a more detailed understanding of the distribution of binding energy within the interfaces of the CEACAM dimer and HopQ-CEACAM complexes, we undertook a comprehensive alanine scanning mutagenesis analysis in which we generated a total of 13 mutants in the IgV domain of CEACAM1 and the extracellular

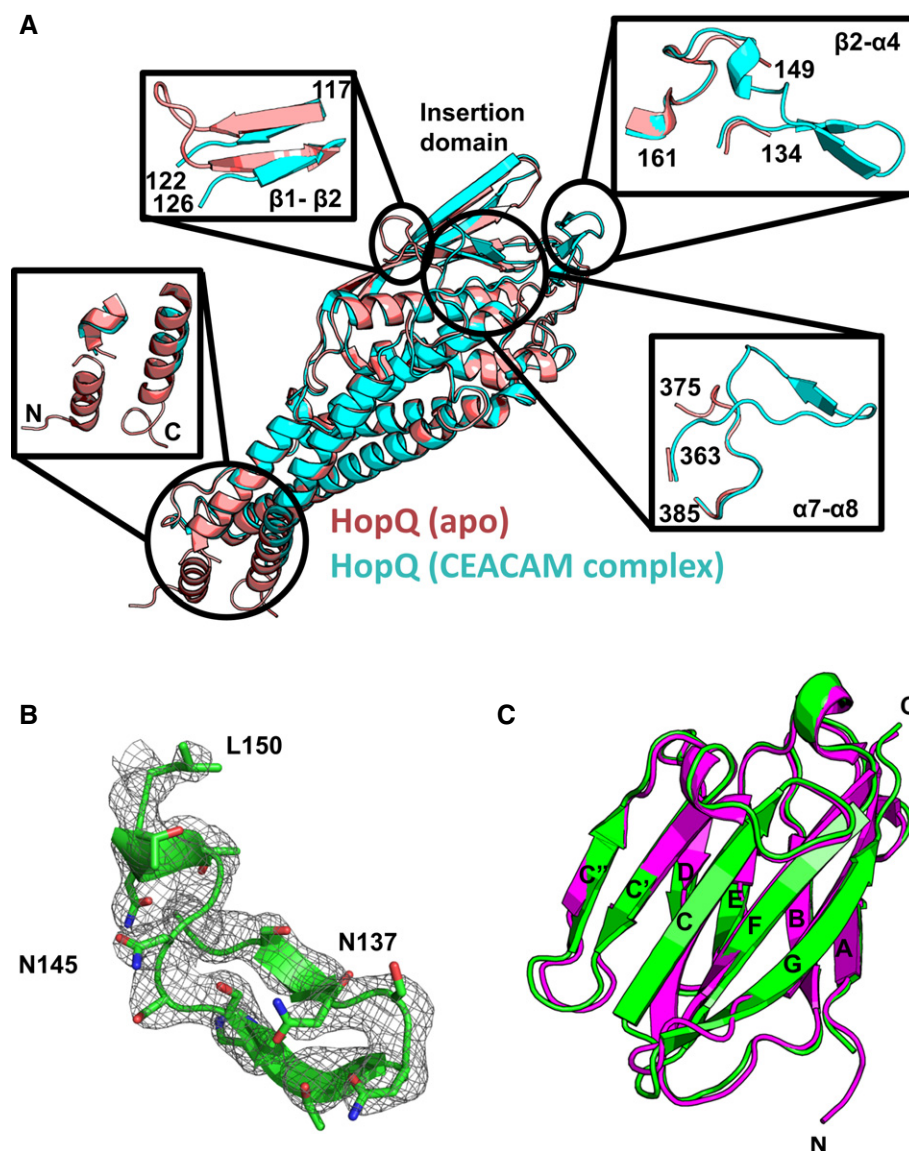


Figure 4. Structural changes of HopQ and CEACAM1 upon complexation.

A Apo-HopQ (pdb code 5lp2, pink) superimposes with HopQ of the HopQ-CEACAM1 complex (cyan) with a RMSD of 0.60 Å. Order-disordering events occur upon complexation including, disordering of the coiled-coils (*bottom left*) and the β1-β2 loop (*top left*), and ordering of the β2-α4 (*top right*) and the α7-α8 (*bottom right*) loops.

B Omit map contoured at 1.2 σ around residues 133–150 of the β2-α4 loop.

C CEACAM1 (pdb code 2gk2, magenta) superimposes with CEACAM1 of the HopQ-CEACAM1 complex (green) with a RMSD of 0.66 Å.

domain of HopQ and measured their effects on CEACAM1 dimerization and on HopQ-CEACAM1 binding, by analytical ultracentrifugation (AUC) and ITC, respectively. This analysis allowed us to quantitatively map and compare the energetic landscapes of both complexes.

Protein–protein interfaces commonly exhibit a distribution of hot spot residues, in which the binding energy of the interaction is highly concentrated, and energetically silent residues. The CEACAM1 homodimer is no exception in this regard, as shown in Figs 5A and EV1, and Table EV2. We observed changes in binding free energy ($\Delta\Delta G$) of up to 3 kcal mol^{−1} for several alanine mutations, including CEACAM1^{F29A}, CEACAM1^{Q44A}, and CEACAM1^{V96A},

and as large as 4 kcal mol^{−1} for the CEACAM1^{N97A} mutation, which effectively converts CEACAM1 into a monomer. Conversely, the CEACAM1^{Q89A} and CEACAM1^{L95A} mutations had little to no effect on CEACAM1 dimerization.

We next tested all of these single-site alanine mutants in CEACAM1 for binding to HopQ. Contrary to CEACAM1 dimerization, we found that none of them had any significant impact on binding affinity, although the thermodynamics of binding did vary with the CEACAM1^{Q89A}, CEACAM1^{L95A}, and CEACAM1^{N97A} exhibiting approximately 8 kcal mol^{−1} less favorable binding enthalpies, which were offset by favorable gains in entropy (Figs 5B—right and EV2, and Table EV2). The variant with the largest effect on the

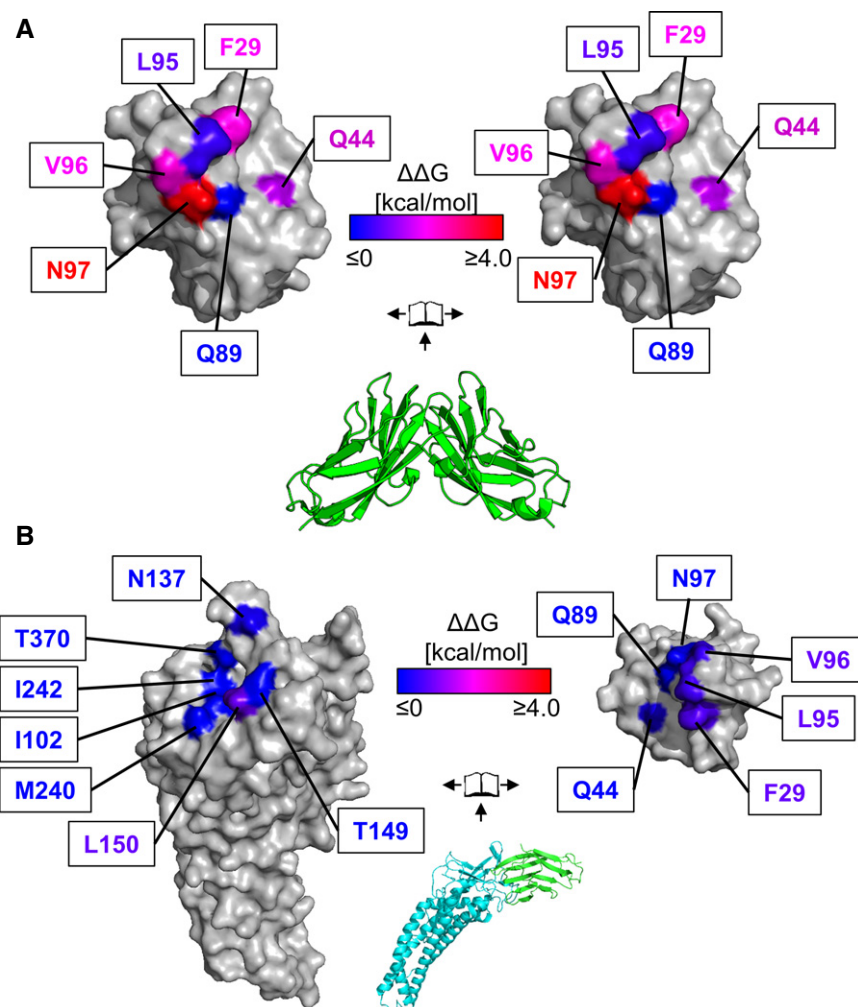


Figure 5. Binding analysis of CEACAM1 homodimerization and complexation with HopQ reveal different binding energies.

A Center—Crystal structure of CEACAM1 homodimer (pdb code 2gk2, green). The dimer is rendered as a molecular surface representation in an open-book conformation (left and right) with the surfaces colored according to changes in binding energies as measured by AUC.

B Center—Crystal structure of HopQ (cyan) bound to CEACAM1 (green). The complex is rendered as a molecular surface representation in an open-book conformation with the surfaces of HopQ (left) and CEACAM1 (right) colored according to changes in binding energies as determined by ITC. Whereas mutations in CEACAM1 cause changes in affinity which span at least a 1,000-fold range in homodimerization, only small differences are observed in complexation with HopQ.

interaction, CEACAM1^{F29A}, bound HopQ only threefold more weakly than did wild-type CEACAM1.

In order to interrogate the HopQ side of the HopQ-CEACAM1 complex interface, we made single-site alanine mutations in HopQ, none of which had a significant effect on HopQ-CEACAM-binding affinities, mirroring the properties of the CEACAM1 alanine mutants (Figs 5B—left and EV2, and Table EV2). Of the seven variants that we were able to express and purify, most exhibited similar affinities to the WT interaction, with only changes in the thermodynamics observed, while a few resulted in only modest resulted affinity changes that, although quantifiable, are unlikely to be physiologically meaningful. One such variant, HopQ^{L150A}, resulted in a modest (~4.5-fold) decrease in affinity (920 nM, Fig EV2J). Seeking a modification of the HopQ-CEACAM1 molecular interface that could dramatically decrease the affinity, we tested HopQ^{Δ135–136Δ141–142}, which completely removes the β -hairpin that

folds in the β 2- α loop. However, these multiple sequence changes again resulted in only a modest (~4.5-fold) reduction in affinity relative to wild-type HopQ (1,000 nM, Fig EV2N). Thermodynamically, this interaction remains exothermic, although the enthalpy of binding is twofold less favorable and is only slightly entropically unfavorable, consistent, in accordance with the comparison of unbound and CEACAM-bound HopQ structures described above, with the folding of the β 2- α loop upon CEACAM1 binding (Fig EV2N). The CEACAM1 Q89A, L95A, and N97A mutations resulted in similar thermodynamic signatures as that of the HopQ^{Δ135–136Δ141–142} mutant. These CEACAM1 residues interact with the HopQ β -hairpin motif in the β 2- α loop (residues 135–138), and thus, either by mutation of these CEACAM1 residues or deletion of the HopQ β -hairpin, we observe a loss of enthalpy and reduction in entropy due to folding of this structural element upon binding.

A sequence alignment of HopQ and BabA reveals two key differences: (i) The BabA insertion domain, which is its recognition domain of glycans, is much larger than that of HopQ; and (ii) the $\beta 2$ - $\alpha 4$ loop in BabA is much shorter than in HopQ and little sequence identity exists between the two (Appendix Fig S2). Several BabA structures have been solved both in the apo form and in complex with Le^b antigens (Hage *et al*, 2015; Moonens *et al*, 2016) and in all of these structures the $\beta 2$ - $\alpha 4$ loop is folded. Several intrinsically disordered prediction programs such as Globplot (Linding *et al*, 2003), IUPred (Dosztanyi *et al*, 2005), RONN (Yang *et al*, 2005), and PONDR (Peng *et al*, 2006), all predict the HopQ $\beta 2$ - $\alpha 4$ loop to be disordered. To test whether this loop in HopQ contributes the majority of the binding energy to the HopQ-CEACAM1 interaction, we constructed a HopQ-BabA chimeric protein, HopQ ^{$\beta 2$ - $\alpha 4$:BabA}, in which the $\beta 2$ - $\alpha 4$ loop of HopQ (residues 133–158) was replaced with that of BabA (residues 136–148). We verified that the HopQ ^{$\beta 2$ - $\alpha 4$:BabA} protein was folded by size-exclusion chromatography and thermal shift assay analyses, but observed that it exhibited no binding to CEACAM1 (Fig EV2O), demonstrating that the $\beta 2$ - $\alpha 4$ loop is specifically recognized by CEACAM1 and contributes substantial binding energy to the HopQ-CEACAM1 interaction.

Molecular determinants of HopQ-CEACAM interaction control CagA translocation

To determine whether the molecular determinants of binding described above also dictate function, we tested our CEACAM1 and HopQ mutants in a functional assay to quantify translocation of the *H. pylori* oncoprotein CagA into human cells. We and others have shown that CagA translocation is dependent on HopQ-CEACAM interactions (Javaheri *et al*, 2016; Koniger *et al*, 2016). First, we transiently transfected HEK293 cells with wild-type or mutant *ceacam1* genes and tested CEACAM1 surface production by FACS analysis (Fig 6A). Next, we infected these cells with *H. pylori* strain P12, producing a TEM-1-CagA fusion protein, or its isogenic Δ hopQ, Δ cagI, or Δ cagT translocation-deficient mutants, and measured TEM-1 β -lactamase activity as a proxy for CagA translocation in an established reporter assay system (Schindele *et al*, 2016; Fig 6B). In agreement with the highly distributed energetic landscape for the HopQ-CEACAM1 interaction, no single-site alanine mutants of *ceacam1* significantly affected CagA translocation relative to wild-type *ceacam1* (Fig 6B).

Second, in order to investigate the role of HopQ mutants in CagA translocation, we complemented *H. pylori* P12[TEM-1-CagA] Δ hopQ with wild-type or mutant *hopQ* genes and confirmed the stable production of recombinant mutant HopQ proteins by immunoblotting (Fig 6C). We then infected HEK293 cells, or HEK293 cells transiently transfected with wild-type *ceacam1*, and quantified CagA translocation by β -lactamase activity. Again, we found that no single-site alanine substitution in *hopQ* had an effect on CagA translocation relative to wild type. The P12 Δ hopQ::*hopQ* ^{$\beta 2$ - $\alpha 4$:BabA} complemented strain, however, was completely defective in CagA translocation, despite the fact that it produced similar amounts of HopQ protein as found for other mutants (Fig 6C and D). We observed similar results when we infected gastric AGS cells with P12 producing the different HopQ variants; in these cells, the *hopQ* mutant is still capable of translocating CagA, albeit at a reduced efficiency (Fig 6E). Together, these data indicate that the molecular

determinants that dictate distinct energetic landscapes in CEACAM dimerization and HopQ-CEACAM interaction translate to the biological function of the HopQ-CEACAM interaction.

Molecular bases of CEACAM self- and HopQ recognition extend to other CEACAMs

Our mutational analysis of CEACAM1 indicates that weakening CEACAM1 dimerization has little to no effect on HopQ binding by CEACAM1. In order to determine whether this held true for other CEACAMs known to bind HopQ and across an even broader range of CEACAM dimerization constants, we constructed a CEACAM IgV domain mutant that resulted in dimerization of an otherwise monomeric CEACAM, CEACAM3, which is found predominantly on phagocytes (Schmitter *et al*, 2004). Indeed, when we analyzed wild-type CEACAM3 by AUC, we found it to be monomeric in solution (Fig EV3A and Table EV2). A Leu-to-Gln mutant at position 44 of CEACAM3, CEACAM3^{L44Q}, resulted in homodimerization with a dimerization constant of approximately 4 μ M (Fig EV3B and Table EV2). We solved the X-ray crystal structures of both wild-type CEACAM3 and CEACAM3^{L44Q} to resolutions of 2.10 and 2.43 Å, respectively (Table EV1). In both of these structures, CEACAM3 packs identically in the respective crystals, even though our AUC data show CEACAM3 to be monomeric in solution (Fig 7A). The structures superimpose with a RMSD of 0.33 Å. These structures reveal that residue Gln44 in the CEACAM3^{L44Q} mutant drives dimerization by forming two hydrogen bonds across the dimerization interface to the backbone of Asn97, one for each pair of residues, that cannot be made by Leu44 in wild-type CEACAM3 (Fig 7A).

Wild-type CEACAM3 binds HopQ with an affinity approximately twofold weaker than does CEACAM1 (Fig EV3 and Table EV2). Despite the dramatic effect that the CEACAM3^{L44Q} mutant has on CEACAM dimerization (i.e., making CEACAM3 a dimer instead of a monomer), it binds with similar affinity to HopQ as does wild-type CEACAM3. In order to determine the structural basis for this discrepancy in driving CEACAM dimerization versus HopQ-CEACAM interaction, we determined the X-ray crystal structures of HopQ in complex with both wild-type CEACAM3 and the CEACAM3^{L44Q} mutant (Fig 7B—left and Table EV1). These structures reveal that although Leu44 and Gln44 reside in the interface with HopQ in the respective structures, the distinct intermolecular contacts made by the mutated residue are not of energetic significance (Fig 7B—right). These data indicate that our findings for CEACAM1 dimerization and HopQ-CEACAM1 interaction can be extended not only to different CEACAMs but also to mutations that either weaken or strengthen CEACAM dimerization.

Discussion

CEACAMs are used by numerous pathogenic bacteria in order to adhere to host cells. The first structures of CEACAMs in complex with a bacterial adhesin reported here clearly show that HopQ binds to the dimerization interface of CEACAMs. This is consistent with all known bacterial adhesins that engage CEACAMs: The Opa proteins from *N. gonorrhoeae* are membrane proteins that use two hypervariable loops to interact with the CEACAM dimerization interface (Billker *et al*, 2000); trimeric UspA1 of *Moraxella*

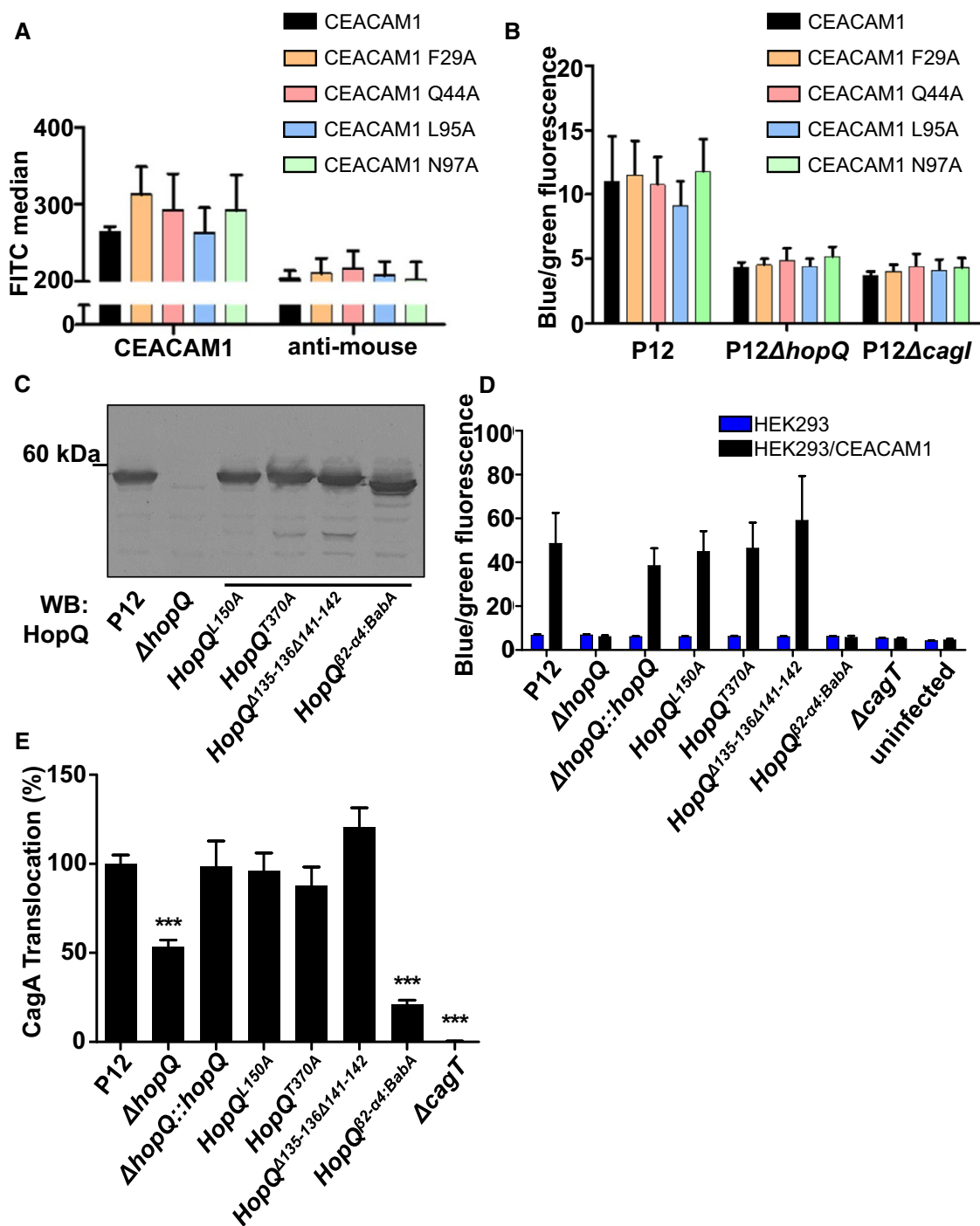


Figure 6. Effects of targeted mutations in HopQ or CEACAM1 for CagA translocation.

A Flow cytometry showing the surface localization of full-length CEACAM1 or derivatives with defined amino acid changes in the interaction domains.
B HEK293 cells transiently transfected with CEACAM1 or CEACAM1 mutant derivatives, infected with P12 wt or mutant *Helicobacter pylori* strains producing TEM-1-CagA. Cells were analyzed using the TEM β -lactamase assay.
C Immunoblot showing the production of wt and mutant HopQ proteins of strain P12.
D HEK293 or HEK293/CEACAM1-producing cells infected with *H. pylori* P12 wt or defined *hopQ* mutant strains producing TEM-1-CagA.
E Gastric epithelial cells (AGS) infected with *H. pylori* P12 wt or mutant strains expressing mutant versions of HopQ.

Data information: The average blue-to-green ratios are shown as absolute values (B and D) or as percentage of the wt strain (E) and represent six independent experiments with standard errors of the mean (with two technical replicates each). Mutations which display significant differences in CagA translocation relative to the P12 wt strain are denoted with *** $P = 0.000001$ for Δ HopQ, *** $P < 0.000001$ for HopQ^{B2- α 4:BabA}, and *** $P < 0.000001$ for Δ cagI. Statistics were calculated with the Mann-Whitney test (two-tailed) using GraphPad Prism. Normal distribution was tested using the Shapiro-Wilk normality test. Data did not follow a normal distribution, so a two-tailed Mann-Whitney test was used to calculate significance. Variation in each group was estimated, and each sample was considered independently.

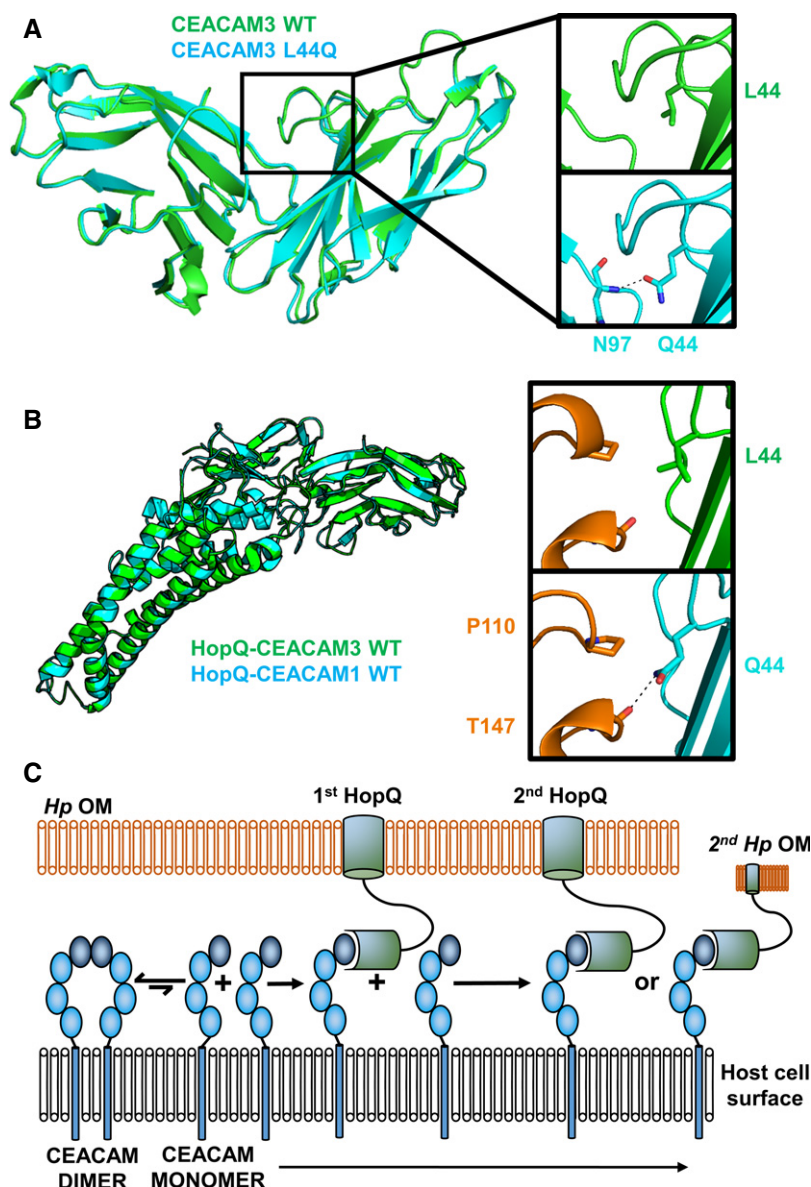


Figure 7. Induced dimerization of CEACAM3 has no effect on binding to HopQ.

- A** CEACAM3 WT (green) at position 44 is a leucine. By AUC, it is monomeric; however, it adopts a dimeric conformation in the crystal structure. CEACAM3 Leu44Gln mutation (cyan) creates two symmetrical hydrogen bonds across the dimer interface to the backbone of Asn97. This causes dimerization of CEACAM3. No structural changes are observed between CEACAM3 and mutant with a RMSD of 0.33 Å.
- B** Superposition of the HopQ-CEACAM1 complex (cyan) with the HopQ-CEACAM3 WT complex reveals little structural changes between the complexes with a RMSD of 0.30 Å. In WT CEACAM3 (green), Leu44 forms van der Waals interactions with Pro110 of HopQ (orange). In CEACAM3, Leu44Gln (cyan) forms van der Waals interactions with Pro110 and a hydrogen bond to the main chain of Thr147 (orange).
- C** Model of HopQ recruitment of CEACAM. CEACAM1 (blue) is predominately dimeric. HopQ (green) binding of CEACAM monomers causes redistribution of dimers to monomers which can bind further HopQ on the same bacterium or recruit other bacteria to the host cell.

catarrhalis contains a 600-Å-long coiled coil, of which a small region close to the OM bends upon binding to the dimerization interface of CEACAM1 (Connors *et al*, 2008); and the Dr adhesins of *E. coli*, which recognize CEACAM5, also bind to the dimerization interface (Korotkova *et al*, 2008). Although the structures of these adhesin proteins are diverse, they all recognize the dimerization interface (CC'C''FG face) of CEACAMs and not the ABDE face. The evolution of adhesion proteins to recognize the dimerization interface may be

due to the presence of conserved glycosylation sites on the ABDE face, which could shield and prevent binding of the adhesins and other proteins.

Prior to our studies, the role of glycosylation in HopQ binding had not been fully explored. HopQ, like SabA and BabA, contains an insertion domain, the role of which in BabA and SabA is to bind Le^b and sLe^x antigens, respectively. It was suggested previously that the HopQ insertion domain was important for binding CEACAM1,

as its deletion reduces the affinity of HopQ to CEACAM1 and a peptide derived from the HopQ insertion domain inhibits the induction of the elongation phenotype of gastric cells when treated with *H. pylori* (Javaheri *et al*, 2016). Our structures, however, clearly show that both CEACAM1 and CEACAM3 do not interact with the insertion domain. Additionally, as the ABDE face is pointing away from the insertion domain and is 30 Å away, it is unlikely that the insertion domain recognizes glycans on CEACAMs as suggested by both our work and that of others (Javaheri *et al*, 2016). However, the importance of the insertion domains of SabA and BabA raises the possibility that a putative function of the HopQ insertion domain is to bind a yet-unknown co-receptor that further facilitates *H. pylori* attachment to host cells.

Numerous molecules expressed by *H. pylori* are important for adherence, including both surface-displayed adhesin proteins (e.g., glycan-binding BabA and SabA, CEACAM-binding HopQ) and proteins that comprise the pilus of the T4SS through which CagA translocates (e.g., β 1-integrin-binding CagL; Barden *et al*, 2014; Bonsor *et al*, 2015b; Bonig *et al*, 2016; Frick-Cheng *et al*, 2016). Our data indicate that HopQ and BabA binding to their respective host cell surface ligands are affected in distinct ways by the chemical environment, including pH. BabA is pH-responsive such that it is released from Le^b glycans in an acidic environment and is able to rebound these ligands upon encountering them again at neutral pH, a property that has been proposed to enable tight mucosal bacterial adherence while providing an effective escape from gastric epithelial cells shed into the acidic gastric lumen (Bugaytsova *et al*, 2017). We show here that HopQ-CEACAM interactions do not exhibit the reversible pH responsiveness that BabA does. Additionally, we previously showed that CagL undergoes a dramatic conformational change in response to pH that affects access to its helical RGD motif required for β 1-integrin binding (Bonsor *et al*, 2015b), providing it a similar pH responsiveness to BabA-Le^b interactions. Collectively, these data suggest a hierarchy of host–pathogen adherence events in which certain steps are reversible, such as bacterial attachment to host cells via Bab-Le^b interactions and even T4SS anchoring via CagL- β 1 integrin interactions, prior to a committed step of HopQ-CEACAM interaction that triggers CagA translocation. Although we have comprehensively described the molecular basis of HopQ-CEACAM interactions, the mechanism(s) by which these interactions trigger or permit CagA translocation remains unclear at this time. It is unlikely to be a signal transduction phenomenon, however, as several CEACAMs with distinct cytoplasmic signaling domains permit CagA translocation, and HopQ has no known signaling properties. It may be primarily a result of the relative high affinities of HopQ-CEACAM interactions, which are in the mid-nM range, that provide sufficient “dwell time” for CagA to be translocated.

Our structures show that CEACAM binds to a HopQ molecular surface composed of several loops of the 3 + 4 helix bundle and that two of these loops fold upon binding. A limited alanine scan of both sides of the binding interface resulted in no clearly defined energetic hot spot, with only a modest threefold to fivefold reduction in binding observed, suggesting that binding energy is dispersed over many residues. We could, however, prevent binding by swapping the β 2- α 4 loop with that of BabA, as measured by ITC analysis and CagA translocation assays. Our data further show that HopQ specifically contacts the dimerization surface of CEACAMs but in a manner that is biophysically distinct from CEACAM homodimerization. Indeed,

our alanine scan of the CEACAM1 dimerization does not cause a dramatic effect on HopQ binding; it does, however, alter the dimerization of CEACAM1 over a 1,000-fold range. Furthermore, we can induce dimerization of CEACAM3 through the Leu44Gln mutation without affecting HopQ binding.

Indeed, recognition of CEACAM monomers by HopQ, as opposed to dimers, may in fact be advantageous for *H. pylori* as it would further induce monomerization of CEACAM dimers due to depletion of the CEACAM monomer pool. This would shift the equilibrium of binding between HopQ and CEACAMs, leading to the binding of additional HopQ molecules on the same *H. pylori*, more firmly anchoring the bacterium to the host cell, and/or allowing adherence to the same host cell by additional *H. pylori* bacteria (Fig 7C).

The HopQ-CEACAM interaction is essential for both host cell adhesion of *H. pylori* and translocation of CagA, making it an attractive target for therapeutic development since inhibitors of this interaction could act as anticancer agents at multiple levels (i.e., as anti-adhesives and anti-oncoprotein translocators). For instance, such inhibitors could be developed by engineering CEACAMs to bind with higher affinity and specificity to HopQ than do wild-type cell surface-expressed CEACAMs. Furthermore, if they were conjugated to antimicrobial agents, such affinity-matured CEACAM variants could function as narrow-spectrum antibiotics to kill *H. pylori* while mitigating damage to commensal bacteria in the gut microbiota.

Materials and Methods

Cloning and mutagenesis of plasmids for bacterial and eukaryotic cell manipulation and expression

Plasmids for expression of full-length CEACAM1 in HEK293 cells and bacterial expression vector of the N-terminal domain of CEACAM1 have been described elsewhere (Koniger *et al*, 2016). The N-terminal domain of CEACAM3 (tagless) was synthesized as a Gene String (Thermo Fisher) and cloned into an *NcoI/XhoI* cut pET21d vector. The extracellular domain of HopQ (residues 38–462, *Hp* strain P12) was cloned into a *BamHI/XhoI*-modified pRSFDuet vector that contains an N-terminal hexahistidine tag for bacterial expression. The ectodomains of CEACAM1 were cloned into the *XmaI/NotI* pSG160 vector with an N-terminal hexahistidine tag following the secretion peptide for expression of glycosylated CEACAM1 in HEK cells. Mutagenesis, loop swaps, and loop deletions were carried out by whole-plasmid mutagenesis (QuikChange Agilent).

Protein expression and purification

The N-terminal domain of CEACAM1, CEACAM3, and mutants was expressed and purified as described before (Koniger *et al*, 2016) with minor modifications. Briefly, BL21(DE3) pLysS cells were grown in 1 l of Miller's Luria Broth to an OD_{600 nm} of ~0.6 at 37°C before induction with 1 mM IPTG. Cells were grown for a further 4 h at 37°C before harvesting. The cell pellet was lysed by sonication in 20 mM Tris–HCl, 500 mM NaCl, pH 7.5 (Buffer A) with 1% v/v Triton X-100. The inclusion bodies (IBs) were isolated by centrifugation, suspended in Buffer A with 1% v/v Triton X-100 and lysed by sonication. The IBs were isolated, suspended in 10 mM Tris, 1.0 M NaCl, pH 8.0, and lysed by sonication. Isolated IBs were

washed with Buffer A, pelleted, and dissolved in ~5 ml 30 mM Tris, 150 mM NaCl, 8.0 M urea, pH 8.3 for 1 h at room temperature. Undissolved material was spun down, and the supernatant was slowly injected (0.1 ml min^{-1}) into 50 ml of 50 mM CHES-HCl, 500 mM L-Arginine, pH 9.2 with stirring at 4°C . The protein was allowed to refold overnight before extensive dialysis against 5 mM Tris, pH 8.0. The protein was concentrated by MonoQ (GE Healthcare). CEACAM1, CEACAM3, and mutants were further purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.5.

HopQ and mutants were expressed in BL21(DE3) pLysS cells and grown in Miller's Luria Broth to an $\text{OD}_{600 \text{ nm}}$ of ~0.6 at 37°C before induction with 1 mM IPTG and growth for 18 h at 18°C . Harvested cells were suspended in 20 mM Tris-HCl, 500 mM NaCl, pH 7.5, and lysed by sonication. Cell debris was removed by centrifugation, and the clarified supernatant was passed over 2 ml of HisPur Ni-NTA resin (Thermo Fisher). The Ni-resin was washed with 25 ml of Buffer A followed by 25 ml of Buffer A with 50 mM imidazole. HopQ was eluted with 10 ml of Buffer A with 400 mM imidazole and further purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.5.

The ectodomains of CEACAM1 were expressed in HEK cells grown in FreeStyle 293 with GlutaMAX and gentamicin (Thermo Fisher) through transfections with the plasmid described above using polyethyleneimine. To produce both glycosylated and non-glycosylated forms of the ectodomains, HEK cells were treated to kifunensine (GlycoSyn, final concentration $1 \mu\text{g ml}^{-1}$) 30 min post-transfection to produce high-mannose glycoproteins. Cells were grown at 37°C with 8% CO_2 . Glycosylated protein was harvested from the supernatant 5 days post-transfection. Non-glycosylated protein was produced by adding EndoA, an endoglycosidase which cleaves high mannose leaving just GlcNAc attached the asparagine, to the cells (final concentration of $1 \mu\text{g ml}^{-1}$) 4 days post-transfections. Non-glycosylated protein was harvest from the supernatant 24 h later. Complex-type glycosylated CEACAM1 ectodomain was produced by not treating the HEK cells with kifunensine or EndoA. Proteins were purified using a 5-ml HisTrap Excel column and further purified on Superdex 200 column (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.5.

Isothermal titration calorimetry

Proteins were extensive dialyzed against 30 mM Tris, 150 mM NaCl, pH 7.5, or 30 mM potassium phosphate, 150 mM NaCl, pH 4.5. ITC experiments were performed using an iTC200 instrument (GE Healthcare). A typical experiment was carried out with the syringe loaded with CEACAM1 (200 μM) and the cell loaded with HopQ (20 μM). Titrations were performed at 25°C with 16 injections of 2.42- μl aliquots, with 230-s intervals between injections. All data were analyzed using Origin 7.0 software.

Thermal shift assays

Proteins were diluted with 20 mM Tris, 150 mM NaCl, pH 7.5 to a final concentration of 10 μM (CEACAM1) or 20 μM (HopQ,

HopQ ^{$\beta 2-\alpha 4$:BabA}, HopQ-CEACAM1, and HopQ ^{$\beta 2-\alpha 4$:BabA}-CEACAM1). One millilitre of protein samples was mixed with 1 μl of 5,000 \times SYPRO Orange. 12.5 μl of each protein-SYPRO Orange was aliquoted to 2×12 wells of a MicroAmp Fast 96-well reaction plate and mixed with 12.5 μl of 200 mM buffer. Twelve buffers were selected to cover pH 2.0–7.5 in 0.5 pH unit intervals; sodium phosphate 2.0 and 2.5; sodium citrate 3.0–6.0; Bis-Tris, pH 6.5; Bis-Tris propane, pH 7.0; and HEPES pH 7.5. The plate was sealed with iCycler Optical Tape, and melting curves were measured from 25 to 95°C on a StepOnePlus Real-Time PCR System. Data were analyzed according to established methods (Niesen *et al*, 2007).

pH effect on the HopQ-CEACAM1 interaction by ELISA

CEACAM1_{IgV} ($1.5 \mu\text{g ml}^{-1}$) in PBS was used to coat a 96-well immunoplate (Nunc MaxiSorp) overnight at 4°C . Wells were blocked with PBS containing 5% w/v non-fat dry milk powder (RPI) and 0.5% w/v BSA (Thermo Fisher Scientific) for 1 h at room temperature. 90 μl of 50 mM buffer system (sodium phosphate pH 2.0; sodium citrate pH 3.0, 4.0, 5.0 and 6.0; Bis-Tris propane pH 7.0) containing 0.5% w/v BSA and 0.05% v/v Tween-20 were added to wells, followed by 10 μl of HopQ ($100 \mu\text{g ml}^{-1}$) and mixed. Plates were incubated at room temperature for 1 h before the addition of either 100 μl of 200 mM of buffer system containing 0.5% w/v BSA and 0.05% v/v Tween-20 or 200 mM Bis-Tris propane, pH 7.0, 0.5% w/v BSA, and 0.05% v/v Tween-20 to read-just the pH to 7.0. Plates were incubated for a further 30 min before removal and washing three times with PBS containing 0.5% w/v BSA and 0.05% v/v Tween-20 (PBSTB). Plates were incubated with 1:5,000 dilution of anti-His HRP conjugate (Merck Millipore) in PBSTB with 5% w/v non-fat milk powder for 1 h at RT before a further three washes with PBSTB. For detection, 1-Step Turbo TMB-ELISA Substrate Solution was used (Thermo Fisher Scientific). Enzymatic reactions were typical stopped 15–30 min later with 1 M sulfuric acid. Absorbances were measured at 450 nm on a Specta-MaxM2 plate reader (Molecular Devices).

Analytical ultracentrifugation measurements

Measurements were performed essentially as described in Bonsor *et al* (2015a). Briefly, sedimentation equilibrium measurements of CEACAM1, CEACAM3, and mutants were performed using an Optima XL-I analytical ultracentrifuge fitted with a four-hole An-60Ti Rotor (Beckman Coulter) at 20°C . Proteins were extensively dialyzed against buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl. Protein partial specific volume and solvent density values from the protein amino acid sequences and buffer composition, respectively, were calculated using SedenTerp (<http://bitcwiki.sr.unh.edu>). Samples were prepared at three concentrations (approximately 12, 18, and 30 μM) and loaded into cells equipped with six-hole charcoal-filled epon centerpieces (1.2 cm path-length) with sapphire windows. Centrifugation was carried out at 63,222, 77,495, and 93,220 g, and scans were acquired at 280 nm with a step size of 0.001 and five averages per step. The data were globally analyzed to both single species and monomer-dimer self-association models using the program HeteroAnalysis.

Crystallization and structure determination of CEACAMs and HopQ-CEACAM complexes

The HopQ-CEACAM1 complex was prepared by mixing a twofold excess of purified CEACAM1 over HopQ. The complex was incubated at room temperature for 30 min before concentration using an Amicon Ultra-15 with a 50,000 NMWL membrane allowing the excess CEACAM1 and unbound HopQ to pass through. The retentate was diluted and concentrated three times using 20 mM Tris, 150 mM NaCl, pH 7.5. The complex was subjected to size-exclusion chromatography using a Superdex 200 column (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.5. Complex fractions were pooled, concentrated to 7.8 mg ml⁻¹, and screened against the Wizards I/II/III/IV (Rigaku) screens using a Crystal Gryphon Protein Crystallography System (Art Robbins Instruments). Crystals grew in condition G11 of the Wizards III/IV screen (20% w/v PEG 6000, 0.1 M HEPES-NaOH, pH 7.0, 0.2 M NaCl). Extensive attempts to reproduce the crystals failed, and therefore, crystals were harvested directly from the initial screen drop. Crystals were washed and cryoprotected in mother liquor containing 20% w/v glycerol. Data were collected on beamline 12-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). Data were processed with XDS (Kabsch, 2010) and indexed and converted to structure factors with Aimless. The complex was solved by molecular replacement using MOLREP and the structures of HopQ (PDB entry 5lp2) and CEACAM1 (PDB entry 2gk2) as search models. The complex was refined with REFMAC and rebuilt in Coot (Winn *et al.*, 2011). The final model and structure factors have been deposited to the RCSB PDB with the entry code of 6AW2.

The HopQ-CEACAM3 WT and L44Q complexes were prepared as for HopQ-CEACAM1 and screened against the Wizards I/II/III/IV screens (Rigaku) at 7.7 mg ml⁻¹. Crystals grew in condition G4 of the Wizards I/II screen (20% w/v PEG 8000, 0.1 M MES-NaOH, pH 6.0, 0.2 M calcium acetate). Crystals were optimized and were found to grow in 20% w/v PEG 3000, 0.1 M Bis-Tris-HCl, pH 6.5, 0.2 M calcium acetate, and 1% v/v glycerol. Crystals were washed and cryoprotected with mother liquor containing 20% v/v glycerol. Data were collected on beamline ID23-D at the Advance Photon Source (HopQ-CEACAM3 WT) 9-2 at SSRL (HopQ-CEACAM3 L44Q). Data were processed with XDS and indexed and converted to structure factors with Aimless. The complex was solved by molecular replacement using MOLREP and the structure of the HopQ-CEACAM1 complex as the search model. The complex was refined with REFMAC and rebuilt in Coot. The final models and structure factors were deposited to the RCSB PDB with the entry code of 6AVZ and 6AW3.

CEACAM3 WT and L44Q mutant were dialyzed against 20 mM Tris, 150 mM NaCl, pH 7.5. WT CEACAM3 was screened against the JCSG⁺ screen (QIAGEN) at 5.6 mg ml⁻¹. Crystals grew in condition H2 (1.0 M ammonium sulfate, 0.1 M Bis-Tris-HCl, pH 5.5, 1% w/v PEG 3350). Crystals were not further optimized and were washed and cryoprotected with mother liquor containing 30% v/v glycerol. Data were collected on beamline 12-2 at SSRL, processed with XDS, and indexed and converted to structure factors with Aimless. CEACAM1 (PDB entry 2gk2) was used as a search model in MOLREP, refined with REFMAC, and rebuilt in Coot. The final model and structure factors have been deposited to the RCSB PDB with the entry code of 6AW1. CEACAM3 L44Q crystallized in a

slightly optimized condition (0.8 M ammonium sulfate, 0.1 M Bis-Tris-HCl, pH 5.5, 1% w/v PEG 3350). Crystals were washed and cryoprotected with mother liquor containing 20% v/v glycerol. A dataset was collected in-house using a Rigaku rotating anode Micro-max-007 beam. Images were processed with iMosflm and intensities were converted to structure factors using Aimless. CEACAM3 WT was used as a search model in MOLREP, refined using REFMAC, and rebuilt in Coot. The final model and structure factors have been deposited to the RCSB PDB with the entry code of 6AW0.

Native mass spectrometry

The HopQ-CEACAM1 complex was buffer exchanged against 50 mM ammonium acetate pH 6.8. Native MS was acquired on a SynaptG2 with a nanoESI source (Waters). Samples were sprayed with gold-coated glass capillaries. Acquisition conditions were as follows: capillary voltage 1.3 kV, sample cone voltage 30 V, extraction cone 3 V, source temperature 40°C, trap CE 10 V, and no transfer CE. Data were analyzed with the Masslynx software (Waters).

Helicobacter pylori complementation assays

The effects of specific amino acid exchanges in HopQ on functionality of the Cag type IV secretion system were determined with *H. pylori* P12 Δ hopQ mutants that were complemented with mutated hopQ expressed from the indigenous plasmid pHel12 (see below). For qualitative assessment of CagA translocation using tyrosine phosphorylation assays, hopQ expression plasmids were transformed into an isogenic Δ hopQ deletion mutant described previously (Koniger *et al.*, 2016). For quantitative determination of CagA translocation into target cells, a marker-free derivative of the previously described *H. pylori* P12 strain producing a TEM-1 β -lactamase-CagA fusion (Schindele *et al.*, 2016) was used. This strain was first transformed with a hopQ deletion plasmid (pFS10), generated by cloning 1-kb hopQ upstream and downstream fragments obtained with primers VK57/VK58 or VK59/VK60, respectively (Koniger *et al.*, 2016), together with an rpsL-erm cassette (Rohrer *et al.*, 2012) into pUC18. The obtained P12 Δ hopQ [TEM1-CagA] strain was then used for complementation with the respective hopQ expression plasmids. As negative controls, strains P12 or P12 [TEM1-CagA] were transformed with either a cagT or a cagI deletion plasmid (Fischer *et al.*, 2001) to disrupt the Cag type IV secretion apparatus.

The hopQ expression plasmids used for complementation were designed to integrate into the indigenous plasmid of strain P12 (pHel12). To achieve this, a pSMART-hcKan vector harboring a fragment of pHel12 (Fischer *et al.*, 2010) was amplified by inverse PCR with primers SR53 (5'-GATCGGATCC CCTACAGGTC TCACACATCA-3') and SR54 (5'-GATCGGATCC CCAAACCTTC CCTATGGGCA-3'), thus generating flanking regions for integration into the intergenic region between hpp12_p11 and hpp12_p01 (nucleotides 9192–10072 and 10131–[10225/1]-946, respectively). Between these flanking regions, we inserted a hopQ expression module, consisting of (i) a cat_{GC} chloramphenicol resistance cassette amplified from the shuttle vector pHel2 (Heuermann & Haas, 1998) with primers WS193 (5'-GGGGTACCCG ATCGGCCATA TTGTGTTGAA AC-3') and LH2 (5'-GATCGGATCC ATCCACGTTG AAAATCTC-3'), (ii) the alpA

promoter (Odenbreit *et al*, 1999) including the multiple cloning site from plasmid pIB6 (Bugaytsova *et al*, 2017), and (iii) the *hopQ* gene amplified with primers CE68 (5'-GATCGTCGAC ATGAAAAAAC-3') and CE69 (5'-GATCAGATCT TTTAATACGC GAACACATAA-3'), resulting in plasmid pWS658. Site-specific mutations were introduced into pWS658 by whole-plasmid mutagenesis.

Expression of *hopQ* constructs by *H. pylori* P12 Δ *hopQ* or P12 [TEM-1-CagA] Δ *hopQ* was confirmed by immunoblotting using the polyclonal HopQ antiserum AK298 (Koniger *et al*, 2016).

For testing the influence of HopQ-CEACAM interactions on type IV secretion efficiency, HEK293 cells were transiently transfected with pRc/CMV-CEACAM1 plasmids harboring full-length CEACAM1 or mutated derivatives, using lipofectamine 2000 transfection, as described previously (Koniger *et al*, 2016). Expression of CEACAM constructs was verified by flow cytometry using the anti-CEACAM1 antibody GM-8G5 (Genovac). Transfected cells or untransfected control cells, or alternatively AGS gastric epithelial cells, were infected with *H. pylori* under standard conditions and examined by phosphotyrosine or TEM-1 β -lactamase assays (either in a plate reader or by flow cytometry), as described previously (Schindele *et al*, 2016).

Data availability

The X-ray crystal structures of HopQ-CEACAM1, HopQ-CEACAM3, HopQ-CEACAM3 L44Q, CEACAM3 WT, and CEACAM3 L44Q from this publication have been deposited to the Protein Databank (www.rcsb.org) with the accession codes of 6AW2, 6AVZ, 6AW3, 6AW1, and 6AW0, respectively.

Expanded View for this article is available online.

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Author contributions

DAB and EJS conceived the project and wrote the paper; WF, DB, PLW, and RH edited the manuscript; DAB conducted X-ray crystallography, mutagenesis, and ITC experiments; DD preformed the MS experiments; JW and DB performed the AUC experiments; QZ and BS performed the functional assays; EW, RB, BD, and WF provided reagents; WF, DB, PLW, RH, and EJS supervised research.

Conflict of interest

The authors declare that they have no conflict of interest.

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